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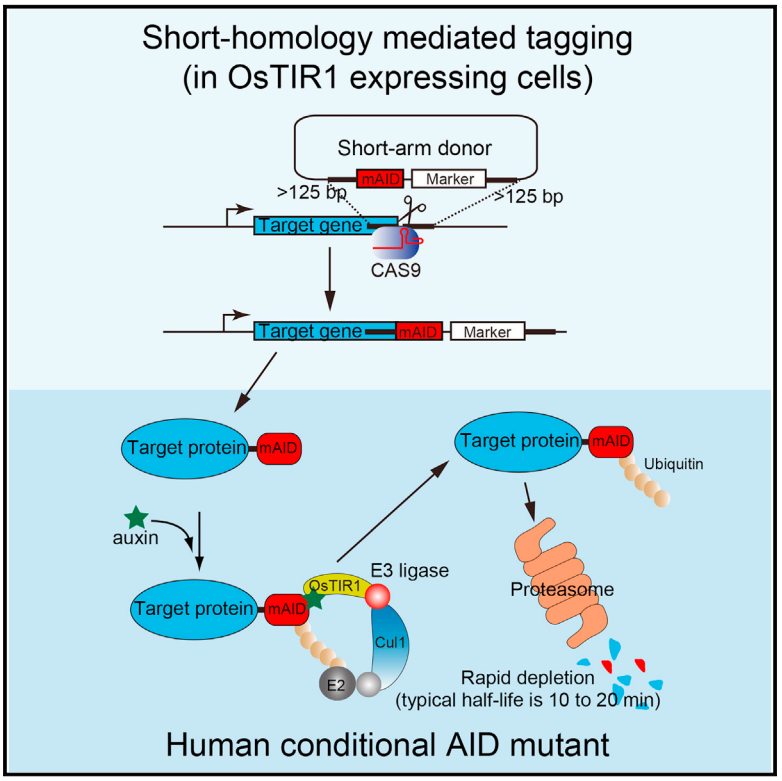
Rapid Protein Depletion in Human Cells by Auxin-Inducible Degron Tagging with Short Homology Donors

Graphical Abstract



Highlights

- Short-arm donors are used for tagging in human HCT116 and mouse ES cells
- Conditional AID mutants are generated in HCT116 cell lines expressing OsTIR1
- Essential proteins fused with AID are depleted rapidly upon the addition of auxin
- The developed method is simple and scalable for construction of AID mutants

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In Brief

Natsume et al. describe a CRISPR/Cas-based tagging method using donor vectors carrying synthetic short homology arms in human HCT116 and mouse ES cells. By tagging the essential *RAD21* and *DHC1* genes in HCT116 cells expressing OsTIR1, the authors generate auxin-sensitive AID mutants in which the target proteins are rapidly depleted.

Natsume et al., 2016, Cell Reports *15*, 210–218
April 5, 2016
http://dx.doi.org/10.1016/j.celrep.2016.03.001

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Rapid Protein Depletion in Human Cells by Auxin-Inducible Degron Tagging with Short Homology Donors

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http://dx.doi.org/10.1016/j.celrep.2016.03.001

SUMMARY

Studying the role of essential proteins is dependent upon a method for rapid inactivation, in order to study the immediate phenotypic consequences. Auxin-inducible degron (AID) technology allows rapid depletion of proteins in animal cells and fungi, but its application to human cells has been limited by the difficulties of tagging endogenous proteins. We have developed a simple and scalable CRISPR/Cas-based method to tag endogenous proteins in human HCT116 and mouse embryonic stem (ES) cells by using donor constructs that harbor synthetic short homology arms. Using a combination of AID tagging with CRISPR/Cas, we have generated conditional alleles of essential nuclear and cytoplasmic proteins in HCT116 cells, which can then be depleted very rapidly after the addition of auxin to the culture medium. This approach should greatly facilitate the functional analysis of essential proteins, particularly those of previously unknown function.

INTRODUCTION

Genetic perturbation is a powerful approach to the analysis of protein function in vivo. The clustered regularly interspaced short-palindromic repeats (CRISPR)/CRISPR-associated (Cas) system-based gene-editing technology has revolutionized the generation of gene knockouts in human cells (Cong et al., 2013; Mali et al., 2013). However, some genes are essential for cellular viability, most of which are involved in processes that are important for proliferation and the number of which is estimated to be ~2,000 in human cell lines (Blomen et al., 2015; Hart et al., 2015; Wang et al., 2015). Because these genes cannot be perturbed constitutively, conditional inactivation or depletion of encoding proteins is useful for the functional analysis.

Conditional depletion can be achieved by fusing a destabilizing domain (so-called degron) that can be conditionally controlled. We previously used heat-inducible degron technology (Dohmen et al., 1994) to study essential budding yeast proteins involved in DNA replication, and we demonstrated that rapid depletion was important to detect the direct consequences of protein inactivation, before the phenotype became obscured by secondary effects (Kanemaki et al., 2003). Several conditional degron technologies, which allow for controlling the stability of degron-fused proteins by use of a small molecule, have been established (Banaszynski et al., 2006; Bonger et al., 2011; Chung et al., 2015; Iwamoto et al., 2010; Neklesa et al., 2011). We developed an auxin-inducible degron (AID) technology by transplanting a plant-specific degradation pathway controlled by a phytohormone, auxin, into non-plant cells (Nishimura et al., 2009). In cells expressing the auxin perceptive F-box protein TIR1, which forms a functional SCF (Skp1–Cullin–F-box) ubiquitin ligase, proteins fused with an AID tag derived from the IAA17 protein of *Arabidopsis thaliana* can be induced for rapid degradation by the addition of auxin to the culture medium. Crucially, AID technology allows for conditional depletion that is very rapid, typically with a half-life of 10–20 min (Holland et al., 2012; Lambrus et al., 2015). This is much quicker than any of the other degron-based technologies that utilize a small molecule, which typically yield half-lives of several hours (reviewed and discussed in Kanemaki, 2013). Furthermore, AID also works in a reversible fashion and has successfully been used for analysis of essential genes in fungi, nematodes, and metazoan cells (Holland et al., 2012; Kanke et al., 2011; Lambrus et al., 2015; Nishimura et al., 2009; Zhang et al., 2015). However, even though the AID system represents a very promising technology for the analysis of essential genes in human cells, its use has been severely limited until now, because it is difficult to fuse endogenous proteins with the AID tag.

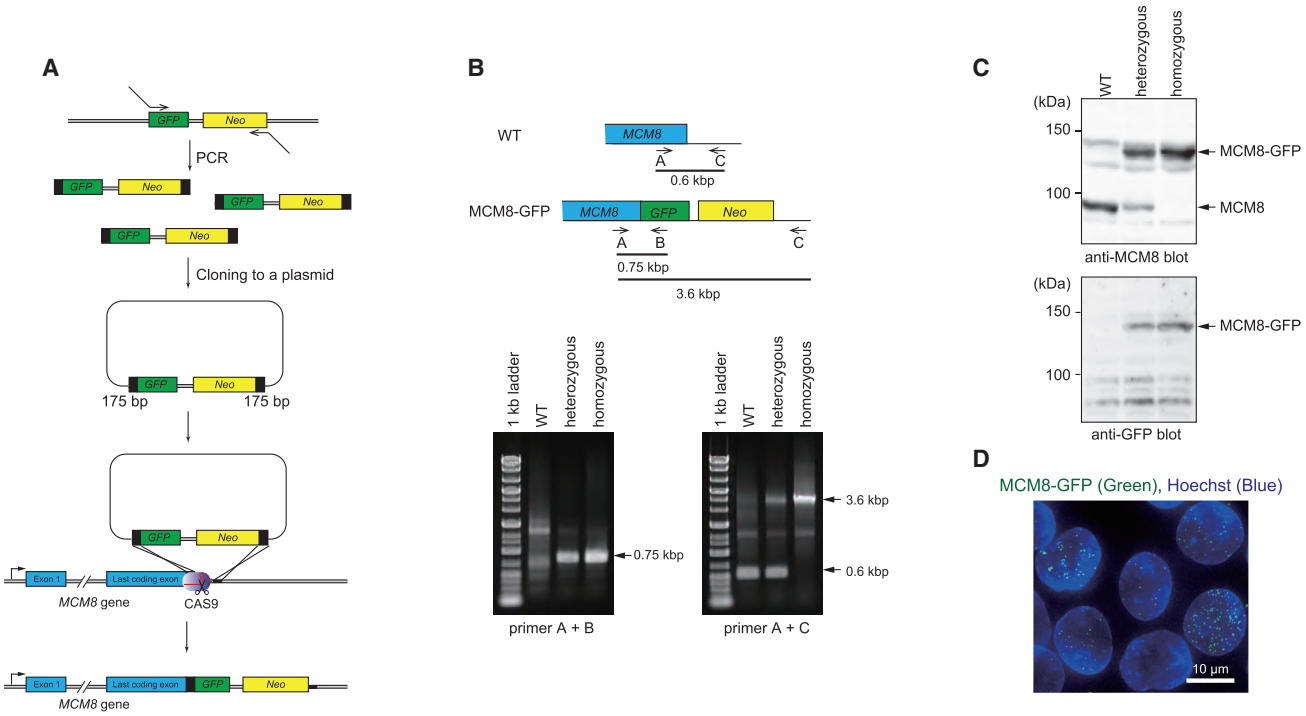

Figure 1. GFP Tagging of the MCM8 Protein Using a Donor Plasmid Harboring Short Homology Arms

(A) Experimental scheme used to tag MCM8 with GFP. A PCR-amplified donor DNA harboring 175-bp homology arms was cloned into pBluescript before transfection with a CRISPR/Cas plasmid to target the MCM8 locus.

(B) Genomic PCR to test the genotype of clones after selection. Primer sets and expected PCR products are shown in the illustration shown above.

(C) The MCM8–GFP protein was detected using anti-MCM8 and anti-GFP antibodies.

(D) Homozygous MCM8–GFP cells were treated with 1 μg/mL of mitomycin C and 10 μM olaparib to induce DNA damage for 24 hr before microscopic observation. MCM8–GFP (green) and Hoechst (blue) staining are shown. The scale bar corresponds to 10 μm.

To overcome this problem, we established a CRISPR/Cas-based method for tagging endogenous genes with donor vectors carrying synthetic short homology arms in human colorectal cancer HCT116 cells, which have a stable diploid karyotype and have been used for conventional gene targeting (Waldman et al., 1995). Here, we describe a simple and scalable method for the generation of conditional human AID mutants of essential genes using donors carrying synthetic short homology arms, by means of a single transfection and subsequent cell cloning. To illustrate the usefulness of this approach, we constructed conditional alleles of the nuclear cohesin and the cytoplasmic dynein complexes, and we demonstrated that AID-tagged RAD21 cohesin subunit was depleted with a half-life of 17 min after the addition of auxin. Finally, we show that tagging with short-homology donors also works in mouse embryonic stem (ES) cells, suggesting that the same strategy can be applied for construction of conditional AID mutants of mouse ES cells.

RESULTS

Gene Tagging with Donors Harboring Synthetic Short Homology Arms

We initially wished to establish a simple CRISPR/Cas-based tagging method to tag endogenous genes of interest before construction of conditional AID mutants. Using CRISPR/Cas, PCR-amplified linear donors harboring short homology arms have been used successfully for tagging endogenous genes in *Drosophila* S2 cells and in nematodes (Böttcher et al., 2014; Paix et al., 2014). We tested this strategy by fusing GFP at the C terminus of the MCM8 protein using a PCR-amplified linear DNA carrying 175-bp homology arms as a donor for homology-directed repair (HDR) (Figure S1A). We found that insertion of the GFP cassette was rarely observed (Figure S1B) and that the MCM8-GFP protein was not expressed in any of the clones obtained (data not shown). We suspected that the linear donor DNA might be unstable in cells because of degradation by exonucleases. Therefore, we cloned the PCR product into a plasmid and used it as a donor for HDR (Figure 1A). After selection with G418, we isolated clones and checked the insertion of the GFP cassette by genomic PCR (Figures 1B and S2). We found heterozygous or homozygous insertion in up to 24% of G418-resistant clones (n = 28 and 33). The expression of the MCM8-GFP fusion protein was confirmed further by immunoblotting (Figure 1C). The MCM8-GFP protein formed nuclear foci upon DNA damage, as shown previously in chicken DT40 cells (Figure 1D; Nishimura et al., 2012), suggesting that the fusion protein is functional. To determine the minimum length of homology arms that is required for HDR in HCT116 cells, we tested similar donor vectors harboring 80-, 125-, 150-, or >740-bp homology arms (Figure S2). Surprisingly, even in

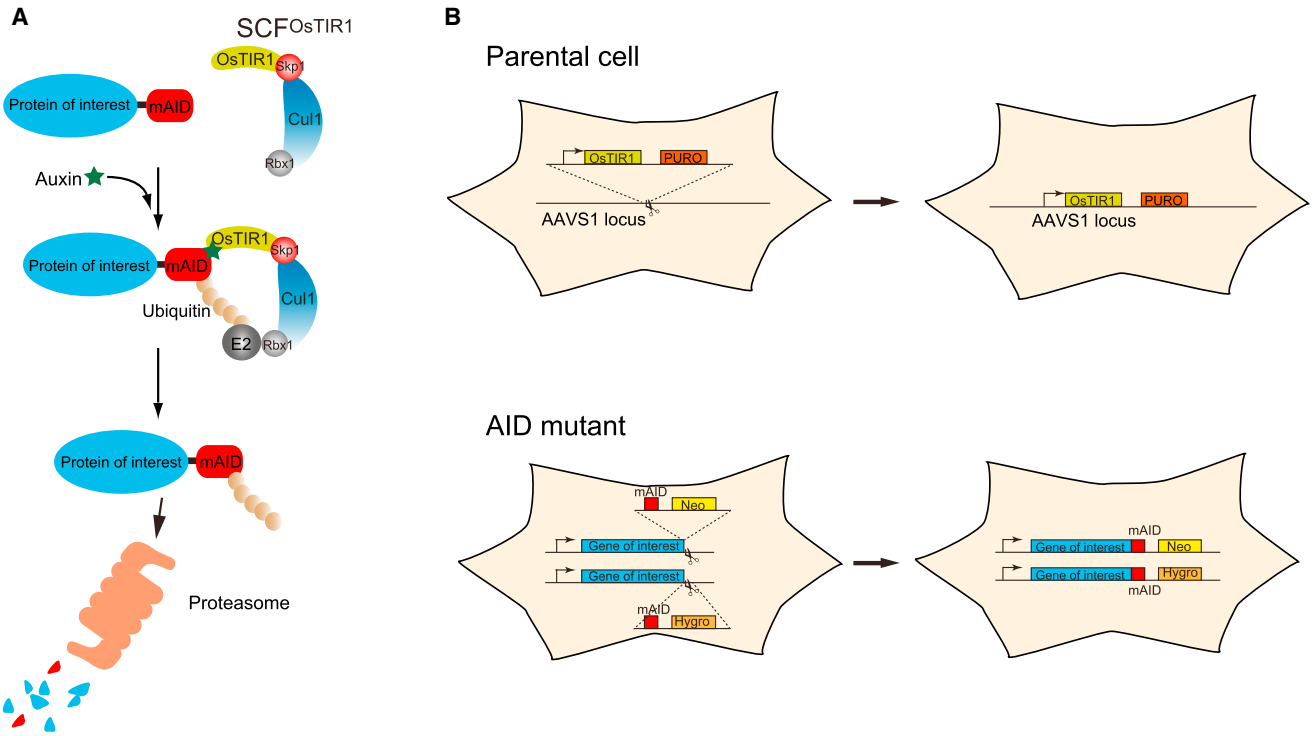

Figure 2. Construction of Human Conditional AID Mutants

(A) A schematic illustration of the AID system. Expressed OsTIR1 can form a functional SCF^{OsTIR1} E3 ligase complex with the endogenous components in human cells (Nishimura et al., 2009). In the presence of auxin, a protein of interest fused with mAID is rapidly degraded upon poly-ubiquitylation.

(B) A schematic illustration showing the strategy of construction of human conditional AID mutants. Initially, parental cells have to be generated by introducing a transgene encoding OsTIR1 at the safe-harbor AAVS1 locus. By tagging of the both alleles of a target gene of interest with mAID in the parental cells, conditional AID mutants can be generated.

the case of 125-bp homology arms, the insertion was observed in 22% of G418-resistant clones (n = 36). Conversely, the donor containing 80-bp homology arms did not work (n = 26 and 35). These results suggest that the minimum homology length required for HDR is 125 bp in HCT116 cells.

Parental Cells for Construction of Conditional AID Mutants

The construction of conditional AID mutants is dependent upon the introduction of two components into cells. The first component is the auxin responsive F-box protein, TIR1, which forms a functional SCF^{TIR1} ubiquitin ligase with the endogenous subunits conserved in all eukaryotic cells (Figure 2A). In this study, we used TIR1 derived from *Oryza sativa* (OsTIR1), because it worked better than its homolog in *A. thaliana* at higher temperatures (Nishimura et al., 2009). The second component is the AID tag that is fused to the protein of interest at the endogenous locus. We used a 68-aa fragment of the original AID/IAA17 tag termed mini-AID (mAID), the molecular weight of which is 7.4 kDa (Figure 2A; Kubota et al., 2013; Nishimura and Kanemaki, 2014).

To construct conditional human AID cells, we took a two-step approach (Figure 2B). We initially generated parental cells by introducing an expression vector encoding OsTIR1 at the safe harbor AAVS1 locus using CRISPR/Cas (Mali et al., 2013; Smith et al., 2008). Subsequently, an in-frame mAID cassette was introduced by CRISPR/Cas into the parental cell line, after the last codon of the gene of interest. We co-transfected two short homology donor vectors containing neomycin (Neo) and hygromycin (Hygro) resistance markers, in order to tag both alleles simultaneously by selecting cells in the presence of G418 and Hygro (Figure 2B; Park et al., 2014). In this way, it is possible to generate conditional AID mutants by a single transfection and subsequent cloning of the parental cell line.

We generated two parental lines that expressed OsTIR1 under the control of the constitutive cytomegalovirus (CMV) or conditional tet promoter (CMV-OsTIR1 or tet-OsTIR1, respectively)(Figures 3A and 3D). After selection with puromycin, the genotype was checked by genomic PCR (Figures 3B and 3E). Subsequently, the expression of OsTIR1 was confirmed by immunoblotting (Figures 3C and 3F). We used a stable clone that carried a homozygous insertion for the subsequent studies. To test whether the expression of OsTIR1 and/or the addition of auxin would cause a side effect, we looked at cell growth and cell-cycle profile of WT and CMV-OsTIR1 cells in the presence or absence of auxin (Figure S3). Both WT and CMV-OsTIR1 cells grew similarly in all culture conditions (Figure S3A). Furthermore, the cell-cycle profile of the cells with or without auxin was also similar (Figures S3B and S3C). These results were consistent with our previous findings that neither OsTIR1 expression nor

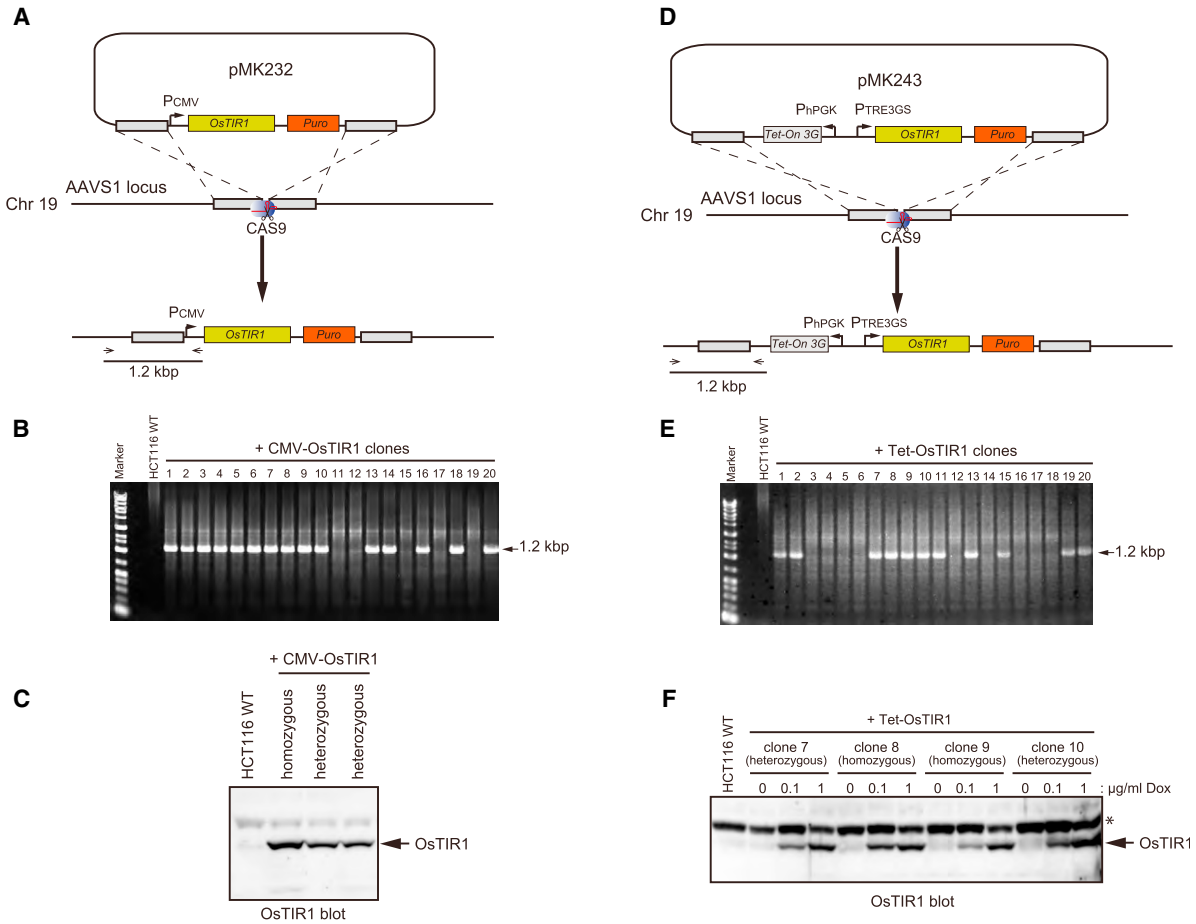

Figure 3. Construction of Parental HCT116 Cells Expressing OsTIR1

(A) Experimental scheme used to introduce CMV-OsTIR1 at the AAVS1 locus. Cells were transfected with pMK232 (AAVS1-CMV-OsTIR1-PURO-AAVS1) and AAVS1-T2 CRISPR/Cas to target AAVS1 locus (Mali et al., 2013). After integration at the AAVS1 locus, the PCR primers should give rise to 1.2 kb products.

(B) After selection with 1 μg/ml puromycin, 20 clones were checked by genomic PCR.

(C) Immunoblotting to detect OsTIR1. The indicated clones were checked by immunoblotting using an anti-OsTIR1 antibody.

(D) Experimental scheme used to introduce tet-OsTIR1 at the AAVS1 locus. Cells were transfected with pMK243 (AAVS1-tetOsTIR1-PURO-AAVS1) and AAVS1-T2 CRISPR/Cas to target AAVS1 locus (Mali et al., 2013). After integration at the AAVS1 locus, the PCR primers should give rise to 1.2 kb products.

(E) After selection with 1 μg/ml puromycin, 20 clones were checked by genomic PCR.

(F) Immunoblotting to detect OsTIR1. The indicated clones were grown in the presence of the indicated concentrations of doxycycline for 24 hr before immunoblotting using an anti-OsTIR1 antibody. The asterisk indicates a background band.

auxin addition significantly affected the expression profile of mRNA in chicken DT40 cells (Nishimura et al., 2009). We concluded that the expression of OsTIR1 and the addition of auxin do not cause a noticeable side effect under our experimental condition.

Conditional AID Allele of the Nuclear Cohesin Complex

To illustrate the efficacy of the AID system for nuclear proteins in human cells, we targeted the RAD21 "kleisin" subunit of the cohesin complex, which is involved in sister chromatid cohesion, DNA repair, and transcription (Jeppsson et al., 2014). Using PCR with long oligonucleotides, we constructed two short-arm donor vectors containing mAID and monomeric Clover (mClover) with a Neo or Hygro resistance marker (Figure 4A; Lam et al., 2012). The two donors were mixed and transfected together with a CRISPR/Cas vector, to insert the AID cassette after the last codon of the *RAD21* gene in wild-type and CMV-OsTIR1 cells. After double selection with G418 and Hygro, we isolated clones and checked the insertion by genomic PCR (Figure 4B). We found that more than 70% of clones that were resistant to G418 and Hygro showed homozygous insertion of the AID cassette at the correct locus, and we confirmed that these cells expressed RAD21-mAID-mClover (RAD21-mAC) in the nucleus (Figure S4).

After treating cells with auxin, we found that the RAD21-mAC protein was depleted very efficiently, specifically in cells expressing OsTIR1 (Figure 4C). Importantly, the auxin-induced depletion of RAD21-mAC was very rapid, with a half-life of 17 min (Figures 4D and S4). Correspondingly, cells exhibited immediate growth arrest upon depletion of RAD21-mAC (Figure 4E). In contrast,

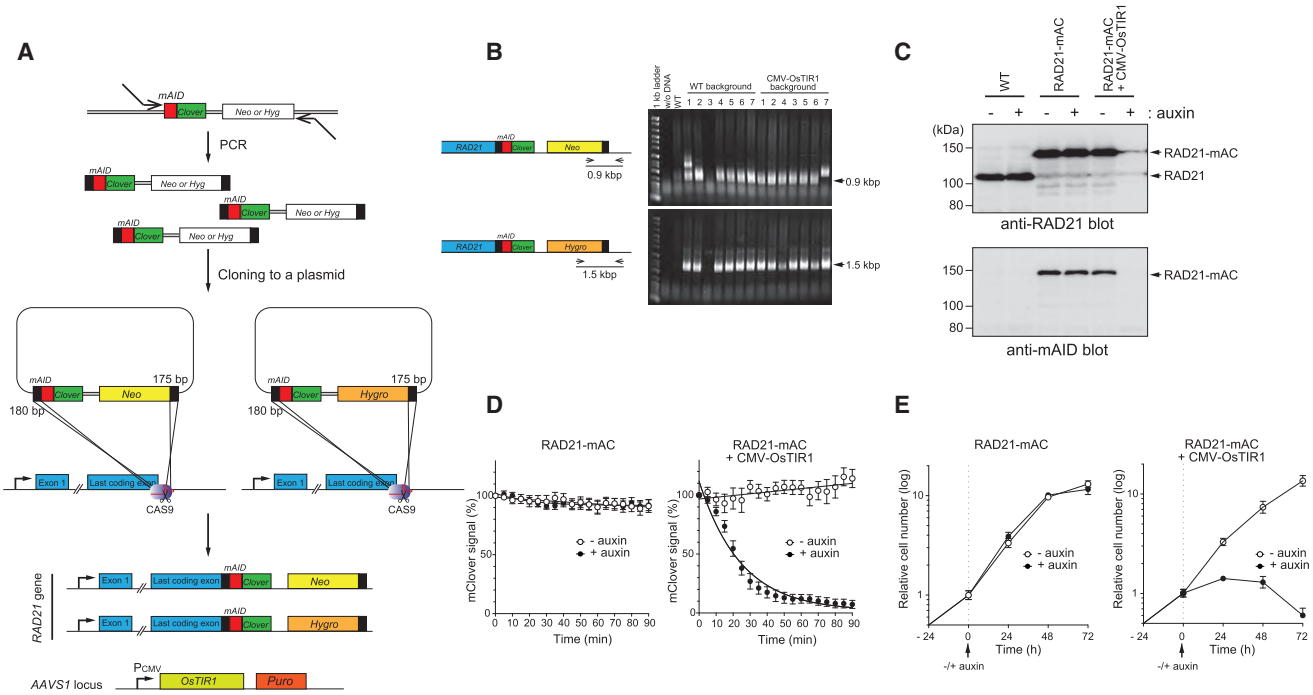

Figure 4. Conditional AID Mutant of RAD21, the Kleisin Subunit of the Cohesin Complex

(A) Experimental scheme used to generate RAD21-mAC cells. A PCR-amplified DNA carrying 180- and 175-bp homology arms was cloned into pCR-BluntII-TOPO. Donor vectors harboring Neo or Hygro were mixed before transfection with a CRISPR/Cas vector to target the RAD21 locus.

(B) Genomic PCR to test the genotype of clones after G418 and hygromycin selection. Primer sets and expected PCR products are shown in the illustration shown on the left.

(C) Detection of the RAD21-mAC protein using anti-RAD21 and anti-mAID antibodies. The indicated cells were treated with or without 500 μM of IAA for 24 hr before protein extraction.

(D) mClover signals were quantified after the addition of 500 μM of IAA (+ auxin) or DMSO (− auxin) by capturing time-lapse images at the indicated time points.

(E) The indicated cells were treated with 500 μM of IAA (+ auxin) or DMSO (− auxin) at 0 hr. The number of cells was counted at the indicated time points.

the growth of RAD21-mAC cells without OsTIR1 was unaffected, which indicated that auxin itself does not inhibit cell growth. These results showed that, combined with the CRISPR/Cas technology, short-arm donors can be used for the generation of human AID mutants.

AID Tagging Allows Rapid Depletion of the Cytoplasmic Dynein Complex

To show that the AID system can also be used to deplete essential human proteins that are localized in the cytoplasm, we targeted the gene encoding the dynein heavy chain 1 (DHC1) protein. This is one of the main components of the cytoplasmic dynein complex, which carries cargo and is essential for establishing chromosome bi-orientation during metaphase (Firestone et al., 2012; Raaijmakers et al., 2013). We constructed two short homology donor vectors containing mAID-mClover with a Neo or Hygro resistance marker using gene synthesis (Figure 5A). We also constructed similar donor vectors harboring 1-kb homology arms that were cloned from the genome.

We initially transfected the short- or long-arm donors into CMV-OsTIR1 cells, using CRISPR/Cas to insert the AID cassette after the last codon of the *DHC1* gene. However, very few colonies grew after selection with G418 and Hygro (Figure S5). We noted previously that the level of AID-fused proteins was reduced slightly in yeast and human cells expressing OsTIR1, even in the absence of auxin (data not shown), probably reflecting the basal activity of the SCF^{OsTIR1} E3 ubiquitin ligase. To overcome this problem, we transfected the same DHC1 donor and CRISPR/Cas vectors into tet-OsTIR1 cells under the repressive condition. After selection with G418 and Hygro, many colonies were formed (Figure S5), regardless of whether we used short- or long-homology arms. In both cases, more than 86% of the isolated clones carried a homozygous insertion (Figure 5B), which indicates that short-arm donors work as efficiently as long-arm donors. We also confirmed that most G418 and Hygro resistant clones exhibited arrested growth in the presence of doxycycline (dox) and auxin, which suggests that in-frame insertion occurred very efficiently (data not shown). We used a clone that was generated using the short-arm donors for subsequent analysis.

We added dox and auxin to induce the expression of OsTIR1 and the degradation of DHC1-mAIC-mClover (DHC1-mAC), respectively, followed by the collection of time-course samples. Immunoblotting showed that OsTIR1 expression was induced from 2 hr and reached a plateau at 10 hr (Figure 5C). Correspondingly, the DHC1-mAC protein was depleted. Considering that the molecular weight of DHC1 is 530 kDa, this result showed that very large proteins can be efficiently depleted using AID

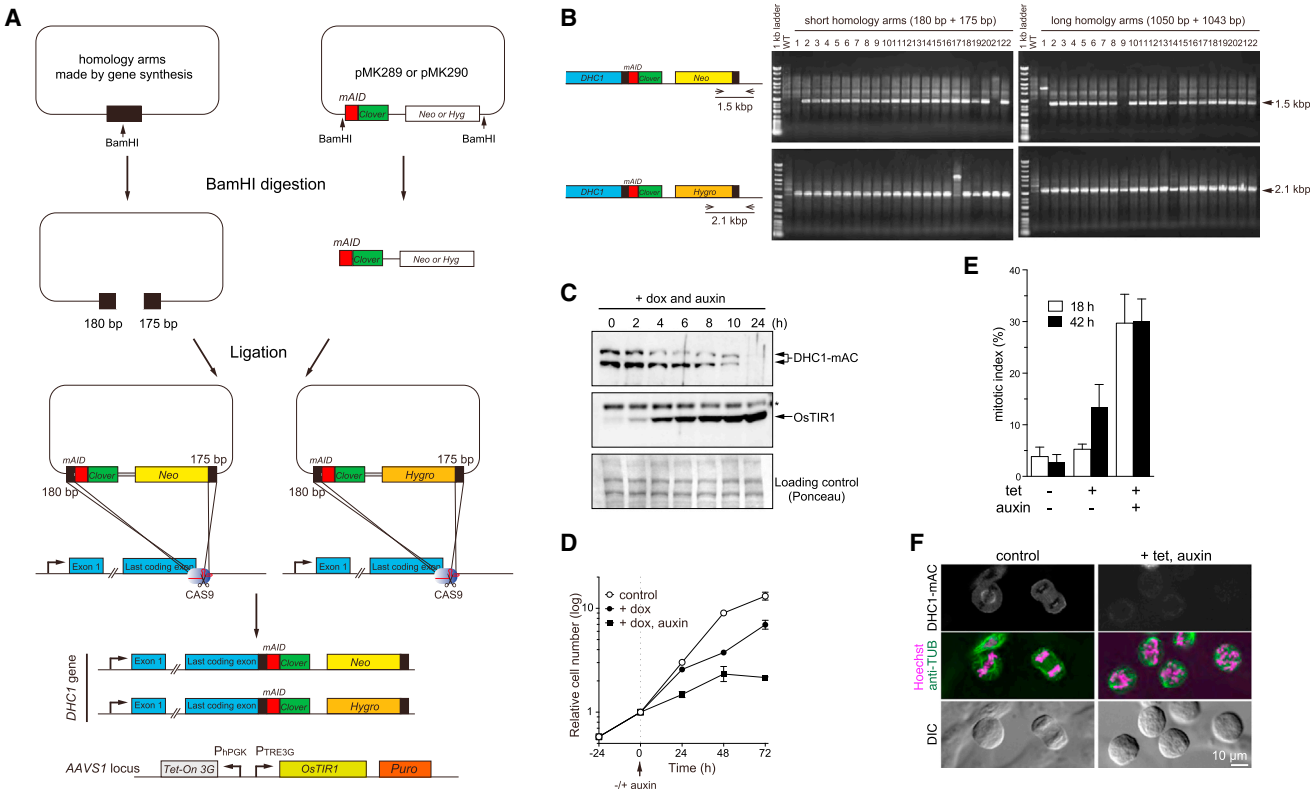

Figure 5. Conditional AID Mutant of DHC1, the Main Subunit of the Cytoplasmic Dynein Complex

(A) Experimental scheme used to generate DHC1-mAC cells. Two donor plasmids harboring 180- and 175-bp homology arms were constructed using gene synthesis and sub-cloning. The donors were mixed before transfection with a CRISPR/Cas vector to target the DHC1 locus.

(B) Genomic PCR to test the genotype of clones after G418 and hygromycin selection. Primer sets and expected PCR products are shown in the illustration shown on the left.

(C) Detection of DHC1 and OsTIR1 using anti-DHC1 and anti-OsTIR1 antibodies, respectively. DHC1-mAC cells were treated with 2 μg/ml doxycycline and 500 μM of IAA.

(D) DHC1-mAC cells were treated with 2 μg/ml doxycycline or 2 μg/ml doxycycline and 500 μM IAA at 0 hr. The number of cells was counted at the indicated time points.

(E) DHC1-mAC cells were treated with 1 μg/ml tetracycline and 500 μM IAA. Mitotic cells with condensed chromosomes and a round cell shape were counted at 18 and 42 hr (n > 500).

(F) The DHC1-mAC cells used in Figure 5E were fixed at 18 hr and stained with an anti-tubulin antibody (green) and SiR Hoechst (magenta) before observation. The scale bar represents 10 μm.

technology. We then analyzed cell proliferation after the addition of dox, or dox and auxin (Figure 5D). In the presence of dox and auxin, the cells exhibited completely arrested proliferation at 48 hr. We noted that the cells treated with dox alone also showed slowed proliferation, although the effect was not as strong as that observed in cells treated with dox and auxin. This was consistent with the notion that SCF^{OsTIR1} somewhat reduced the expression level of AID-fused proteins, even without auxin, and with our finding that DHC1-mAC cells were not generated in the CMV-OsTIR1 background. We conducted a similar experiment using tetracycline (tet) instead of dox, and we checked chromosomes and cell morphology (Figures 5E and 5F). After the addition of tet and auxin, mitotic cells accumulated, in which chromosomes were misaligned. This phenotype was consistent with previous findings of loss of DHC1 function by small interfering RNA (siRNA) or inhibitor treatment, which caused chromosome misalignment and mitotic arrest (Firestone et al., 2012; Raaijmakers et al., 2013). Overall, these findings indicated that conditional AID mutants can be generated in human cells for essential nuclear and cytoplasmic proteins, using synthetic short-arm donors.

Tagging with Short-Arm Donor Works in Mouse ES Cells

To test whether the CRISPR-based tagging with short-arm donors works in other cell types, we tagged the *MCM2* gene in mouse ES cells, which have been used for the conventional gene targeting (Thomas and Capecchi, 1987). We constructed a donor vector carrying mAID-mClover, a Neo-resistant marker, and 200-bp homology arms by gene synthesis and one-step cloning as described in Figure 5A. The donor vector was transfected into mouse ES cells together with a CRISPR/Cas vector for targeting the C-terminal coding region of the *MCM2* gene (Figure 6A). After selection by G418, colonies were isolated for genotyping by PCR. We found that at least one allele of the

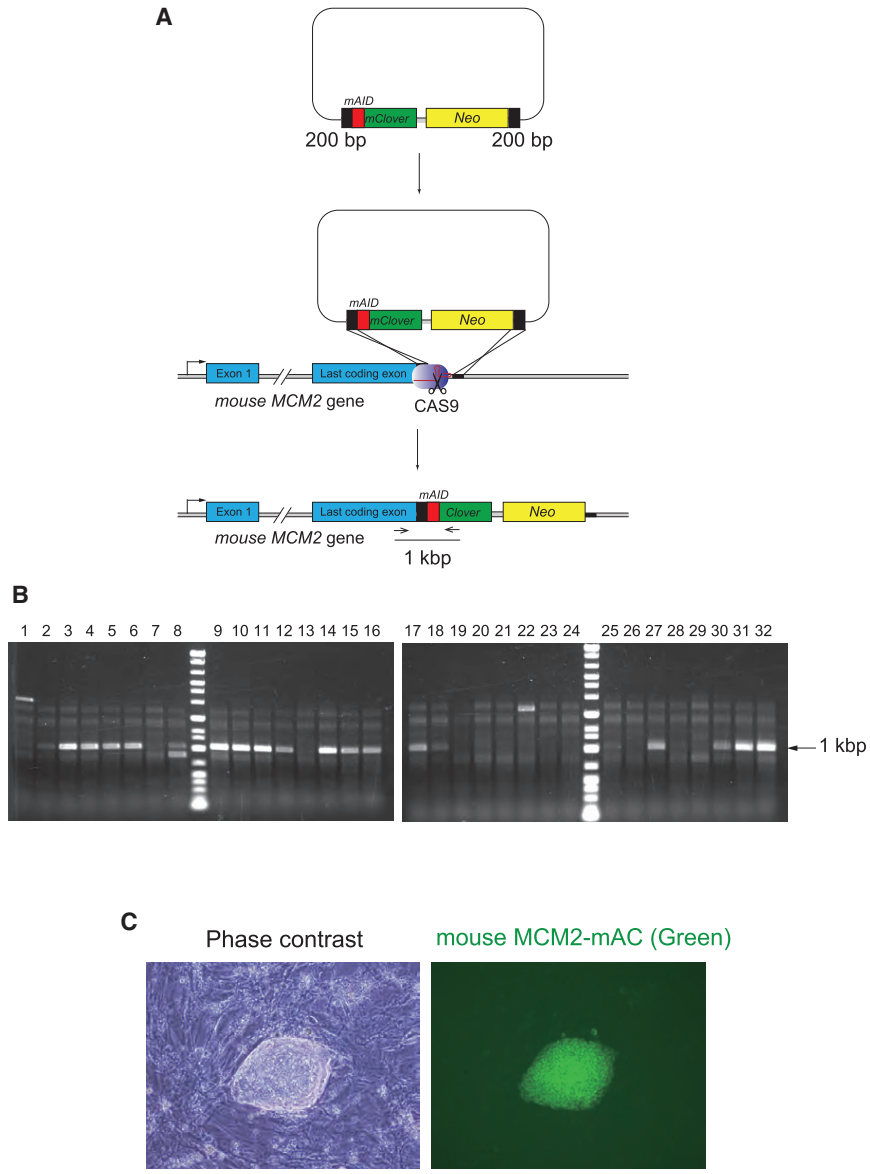

Figure 6. mAID-mClover Tagging of the MCM2 Protein Using a Donor Plasmid Harboring Short Homology Arms in Mouse ES Cells

(A) Experimental scheme used to tag MCM2 with mAID-mClover. A donor plasmid harboring 200-bp homology arms with a neomycin maker was constructed using gene synthesis and sub-cloning. The donor was transfected with a CRISPR/Cas vector to target the MCM2 locus.

(B) Genomic PCR to test the genotype of clones after G418 selection. Primer sets and expected PCR products are shown in (A).

(C) Microscopic observation of a positive colony. MCM2-mAID-mClover (MCM2-mAC) localized in the nucleus was detected under a fluorescent microscope.

MCM2 gene was tagged in 50% of G418-resistant clones (n = 32) (Figure 6B). Consistent with this result, we noted that ~50% of colonies were mClover positive (Figure 6C; data not shown). Based on these results, we conclude that tagging with short-arm donors efficiently works even in mouse ES cells and suggest that AID conditional mutants of mouse ES cells can be generated following a similar strategy described in this paper.

DISCUSSION

In this report, we showed that it is possible to tag endogenous genes in human HCT116 and mouse ES cells, using donor vectors harboring short homology arms. As shown in Figure S2, the minimum homology length required for tagging MCM8 in HCT116 was 125 bp. However, the minimum length might vary depending on the locus of the gene of interest. Using 175- to 220-bp homology arms, we have successfully tagged more than ten endogenous proteins in HCT116 cells to date. To place fluorescent, purification, or mAID tags at the C terminus of a protein of interest, we generated a series of plasmids for the construction of donor vectors, using PCR or via simple cloning after gene synthesis (Figure S6).

We used two types of parental HCT116 cells in which OsTIR1 expression was driven by the constitutive CMV or the conditional tet promoter (Figure 3). As typified by DHC1 (Figure S5), there are cases in which we have been unable to generate AID mutants in the CMV-OsTIR1 background, possibly because of the reduced expression of the AID-fused protein, due to the basal activity level of CMV-expressed OsTIR1. Therefore, it will be helpful to test both parental cell lines for the generation of new AID conditional mutants. The addition of auxin induces the rapid degradation of AID-fused proteins, typically with a half-life of 10–20 min (Holland et al., 2012; Lambrus et al., 2015; Nishimura et al., 2009). Consistently, Figure 4D showed that the half-life of RAD21-mAC was 17 min in the presence of auxin. In the case of the tet-OsTIR1 background, an initial period of ~10 hr is required to induce the maximum expression level of OsTIR1, following the addition of either doxycycline or tetracycline (Figure 5C). To deplete AID-fused proteins as quickly as possible when using the tet-OsTIR1 system, pre-induction of OsTIR1 before auxin addition is likely to be helpful.

We used HCT116 cells for knockin experiments because of their high efficiency of HDR and their diploid karyotype (Waldman et al., 1995). Therefore, it is necessary to test whether the short-arm donors are efficient for CRISPR/Cas-mediated HDR in other cell types. We expect that the same tagging strategy can be applied to cell lines that are used for conventional gene targeting, such as human NALM6 cells, chicken DT40 cells,

and other types of cells. In fact, we successfully showed that short-arm-donor-based tagging worked in mouse ES cells (Figure 6). Our tagging method using donors harboring short synthetic homology arms employs the conventional HDR pathway and can be used as an alternative to the recently described tagging method that uses micro-homology-mediated end-joining repair (Nakade et al., 2014). Overall, in combination with the CRISPR/Cas technology, our strategy facilitates gene tagging at endogenous loci and opens the door to the possibility of generating genome-wide conditional AID mutants for all essential genes in human cells.

EXPERIMENTAL PROCEDURES

Plasmid Construction

We used pX330-U6-Chimeric_BB-CBh-hSpCas9 from Feng Zhang (Addgene #42230; Cong et al., 2013) to construct CRISPR/Cas vectors according to the protocol of Ran et al. (Ran et al., 2013). To construct donor plasmids for the expression of OsTIR1 from the AAVS1 locus, AAVS1 hPGK-Puro-pA from Rudolf Jaenisch (Addgene #22072; Hockemeyer et al., 2009) was modified. The CMV and tet promoters from pIRES2-AcGFP (Clontech) and ptet-One (Clontech), respectively, the SV40 terminator from pIRES2-AcGFP, and a codon-optimized *OsTIR1* gene were used. The template plasmids shown in Figure S6A were based on pBluescript and constructed using synthesized DNA and genes encoding mClover and mCherry2 from Michael Lin (Addgene #40259; Lam et al., 2012) and Michael Davidson (Addgene #54517), respectively. An experimental overview of the construction of donor vectors is presented in Figure S6B. To construct donor vectors by PCR (Figures 1A and 4A), the template DNA was amplified using Phusion DNA polymerase (New England Biolabs) and 200-base oligonucleotides containing a 175-base homology sequence to the target locus. Amplified DNA was cloned at the EcoRV site of pBluescript or into pCR Blunt II-TOPO (Thermo Fisher Scientific). To construct donor vectors via gene synthesis (Figure 5A), homology arms that were split by the BamHI sequence were synthesized. The mAID-mClover cassette containing a selection marker was excised by BamHI and cloned at the BamHI site, between the homology arms. In all cases, homology arms were designed to introduce a silent mutation or to delete partly the recognition sequence after integration at the target locus, to prevent re-cutting. All plasmids shown in Figure S6A are available from Addgene (https://www.addgene.org) and the Gene Engineering Division of the National Bioresource Project (http://dna.brc.riken.jp).

Cell Culture, Transfection, and Colony Isolation

The original HCT116 cells were obtained from American Type Culture Collection (ATCC CCL-247) and were cultured in McCoy's 5A medium (Thermo Fisher Scientific) supplemented with 10% FBS (Gibco), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were grown in a 37°C humid incubator with 5% CO_2. To generate stable cell lines, cells were grown in a six-well plate before CRISPR/Cas and donor plasmids were transfected using FuGene HD (Promega). Two days after transfection, cells were removed and diluted in 10-cm dishes, followed by selection in the presence of 1 μg/ml puromycin, 700 μg/ml G418, and/or 100 μg/ml HygroGold. After 10–12 days, colonies were picked for further selection in a 96-well plate. To induce the degradation of AID-fused proteins, 500 μM indole-3-acetic acid (IAA), a natural auxin, was added to the culture medium. In Figure 6, mouse TT2 ES cells were used following the culture method as previously described (Yagi et al., 1993). After transfection, ES cells were selected in the presence of 300 μg/ml G418 for 8 days. Detailed methods for transfection, selection, and colony isolation of human HCT116 and mouse ES cells are described in Supplemental Experimental Procedures. HCT116 cell lines expressing OsTIR1 are available from the Cell Bank of the National Bioresource Project (http://cell.brc.riken.jp/en/).

Genomic PCR

To prepare genomic DNA, cells were lysed in lysis solution (100 mM Tris-HCl [pH 8.0], 200 mM NaCl, 5 mM EDTA, 1% SDS, and 0.6 mg/ml proteinase K), followed by incubation at 55°C for 1 hr. After isopropanol precipitation, DNA pellets were washed in 70% ethanol before re-suspension in TE containing 50 μg/ml RNase A. The DNA solution was incubated at 37°C O/N, for degradation of RNA. Genomic PCR was carried out using Tks Gflex DNA polymerase (TaKaRa) according to the manufacturer's instruction and 30 cycles of the following protocol: 98°C, 10 s → 55°C, 15 s → 68°C, 0.5 min/kb.

Protein Detection

To prepare protein extracts, cells were collected and washed with PBS before lysing in RIPA buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS) containing a protease inhibitor cocktail (Complete EDTA free; Roche). Protein concentration was checked using the Bradford assay before adding 2× SDS sample buffer (125 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 0.01% bromophenol blue, and 10% 2-mercaptoethanol). After denaturation at 95°C for 5 min, equal amounts of total protein were separated using SDS-PAGE. Proteins were transferred to a Hybond ECL membrane (GE Healthcare) and blotted with antibodies after blocking in 5% skim milk/TBST for 1 hr at room temperature. Detection was performed using the Amersham ECL Prime reagents (GE Healthcare) with a ChemiDoc Touch system (Bio-Rad). Anti-MCM8 and anti-OsTIR1 polyclonal antibodies raised in-house were used for the detection of MCM8 and OsTIR1, respectively (Nishimura et al., 2012). To detect mAID, RAD21, and DHC1, commercial antibodies (MBL M214-3, MBL K0202-3, and Santa Cruz sc-9115, respectively) were used.

Microscopy

To detect the GFP and mClover signals shown in Figure 1D and Figure S4, respectively, we captured images of live cells using a DeltaVision deconvolution microscope (GE Healthcare) equipped with an incubation chamber and a CO_2 supply system. Cells were cultured in supplemented McCoy's medium without phenol red. To visualize nuclei, 1 μg/ml Hoechst 33342 was added before observation. To detect mClover signals shown in Figure 6C, we used a fluorescent IX70 microscope (Olympus). To acquire the images shown in Figure 5F, cells were fixed with 3% paraformaldehyde and stained with a 500× diluted anti-α-tubulin antibody (T9026, clone DM1a; Sigma-Aldrich) and 50 μM SiR-Hoechst (Spirochrome) before observation under a Nikon Ti-E microscope equipped with a Yokogawa CSU-W1 spinning-disk confocal scanner.

Flow Cytometry

Cells were harvested and fixed in 70% EtOH. Fixed cells were washed and resuspended in PBS containing 1% BSA, 50 μg/ml RNase A, and 40 μg/ml propidium iodide. After incubation at 37°C for 30 min, treated cells were filtered through a nylon mesh filter (pore size 42 μm) for analysis by the Accuri C6 FACS machine (BD) equipped with FCS4 Express Cytometry software (De-Novo Software).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.03.001.

AUTHOR CONTRIBUTIONS

M.T.K., T.N., and T.K. designed and performed the experiments. Y.S. performed cell culture and transfection of mouse ES cells. The manuscript was written by M.T.K. All authors discussed and checked the manuscript.

ACKNOWLEDGMENTS

We thank Akemi Mizuguchi and Fukumi Kamada for experimental support and Prof. Karim Labib (University of Dundee) and Bryan John Subong for critical reading of the manuscript. This work was supported by the JST PRESTO program, JSPS KAKENHI grants (25891026, 15K18482, 21681027, and 25131722), and the NIG Collaborative Research Program (2014-B and 2015-A1).

Received: December 14, 2015
Revised: January 28, 2016
Accepted: February 24, 2016
Published: March 24, 2016

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